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The Integrative Cardiac Health Program (ICHP) aims to understand the complex interactions of numerous molecular components that characterize individuals or populations at risk for cardiovascular disease (CVD). We are working to identify molecular networks that define cardiovascular risk and correspond to lifestyle changes that may influence the trajectory of disease progression. Given the increasing trends of major CVD risk factors in the US military population and potentially devastating consequences on combat readiness, our efforts are directed to (1) better understanding the relationships of war stressors and CVD risk at the molecular level before onset of clinical disease, and (2) outcomes-based patient empowering lifestyle solutions to prevent disease.

WRNMMC ICHP has developed a standardized and personalized lifestyle intervention program that has resulted in significant improvements in cardiovascular risk markers such as C-reactive protein (CRP), glucose, insulin, glycosylated hemoglobin, and lipids. Our previous research was the first to identify significant gene expression changes associated with an ultra-intensive lifestyle change program and to show that genetic variants at genes involved in lipid metabolism influence lipid response. We hypothesize that (1) identifying genetic influences on CVD and integrating information on dietary, behavioral, and lifestyle factors will provide important information on CVD risk reduction and (2) discovering new genes in previously associated pathways will reveal new molecular influences on cardiovascular risk reduction.

This research is using state-of-the-art next-generation DNA and RNA sequencing to address the following research questions:
1. Can inherited variants in specific genes that influence early coronary events such as MI in young people, be identified through whole-genome sequencing of an appropriate population of affected individuals?
2. Does surgically-induced weight loss alter patterns of gene expression in adipose tissue and peripheral blood, and do these molecular changes have prognostic value in predicting weight loss success and improving the effectiveness of treatment programs for obesity?
3. Can changes in whole-transcriptome expression: (a) improve the effectiveness of lifestyle programs by identifying patients who are unlikely to benefit from the conventional program and should follow a customized program tailored to their individual needs; (b) be used to reduce health outcome disparities between men and women and between specific subgroups of patients; and (c) identify previously unknown molecular pathways that influence heart disease, and provide insights into cardiovascular disease development that may have important consequences for cardiac treatment programs?

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1. **INTRODUCTION:** There is an increasing prevalence of obesity and cardiovascular disease (CVD) risk factors in the military population, which is negatively affecting operational readiness. The ability to prevent heart disease and reduce its overall impact on morbidity, would increase the quality of life among military personnel and their dependents, and has the potential to generate enormous cost savings for the DoD. In the Integrative Cardiac Health Program (ICHP), we are investigating physiological and molecular responses to risk factor modification interventions in individuals or populations at risk for cardiovascular disease (CVD). We aim to better understand CVD risk at the molecular level before onset of clinical disease, and develop outcomes-based patient empowering lifestyle solutions to prevent disease. Through this research, our objectives are to (1) identify genetic influences on CVD and integrate information on dietary, behavioral, and lifestyle factors to provide important information on CVD risk reduction and (2) discover new genes in previously associated pathways to reveal new molecular influences on cardiovascular risk reduction.

2. **KEYWORDS:** Lifestyle modification, cardiovascular disease, obesity, gene expression, RNA sequencing, gender differences, molecular response, diet, exercise.

3. **OVERALL PROJECT SUMMARY:**
For all tasks, much effort was devoted to ordering the new equipment necessary to conduct the whole-genome and RNA sequencing activities, contracting electrical work to accommodate these new sophisticated machines, and arranging for installation and training on the new machines. The HiSeq and MiSeq DNA sequencing systems were ordered on 07/24/2012, and received on 08/17/2012 and 08/28/2012, respectively. The MiSeq system was installed on 09/11/2012, and a two-day training course on system operation, software, and quality assurance was completed shortly thereafter. Another piece of equipment necessary for this award, the Agilent Technologies 2100 Bioanalyzer, was ordered on 08/01/2012, received on 09/26/2012, and began operation on 10/12/2012. Installation and training on operation of the HiSeq sequencing equipment was conducted 11/06/2012–11/09/2012. During the training, samples of total RNA and globin-reduced RNA were subjected to next generation sequencing (NGS) as follows: 3 total RNA isolated from adipose tissue, 6 mRNA isolated from PAXgene tubes (1μg and 100 ng), and 6 globin-reduced RNA (1 μg and 100 ng). Although the initial analysis of the RNA samples subjected to NGS during the HiSeq training indicated good quality sequence, further detailed analysis showed good quality over short read-lengths only. Most samples did not pass a number of QC parameters, indicating DNA contamination. Experiments are ongoing to enhance DNA removal from RNA.

Task #1: Whole-genome sequencing to discover genes influencing early coronary events
This task is completely dependent on receiving serum samples from military personnel in the Department of Defense Serum Repository (DoDSR). Although we

had received correspondence from DoDSR personnel indicating willingness to collaborate, we received a letter from Angelia Eick-Cost, PhD, ScM from the Armed Forces Health Surveillance Center on Sep 7, 2012 declining to support this task due to privacy concerns with whole-genome sequence data. We prepared a response letter and returned it to her on Oct 4, 2012 pointing out the importance of the project, outlining current guidelines from the Office for Human Research Protections, which clearly do not prohibit studies such as this, and requesting that the AFHSC staff reconsider their position. We have received no further correspondence from this office. No progress has been made on this task because no samples have been received.

Task #2: Profile metabolic activity in adipose tissue during surgical weight loss
During the year, 68 new patients undergoing laparoscopically placed adjustable gastric banding (LAGB) were recruited and enrolled in the study. All patients provided proper informed consent. Baseline blood and adipose tissue samples were collected from these new participants. Blood samples were collected from LAGB patients at 145 follow-up examinations, ranging from the six-month follow-up to several years after LAGB. In total, 3,197 aliquots of DNA, RNA, and plasma for biomarker assays were collected and stored. Staff collected 350 data sheets on LAGB patients and entered the data (BMI and other measures of adiposity) into our electronic database. Total RNA was isolated from 194 blood samples from 119 patients. The range of RNA concentrations was 6.2 ng/µl – 233.5 ng/µl. Globin-reduction was performed on 165 total RNA samples from blood. RNA was isolated from 45 adipose tissue samples with concentrations of 42.1 ng/µl – 1112.3 ng/µl. The Tru-Seq total RNA sample preparation kit was used to purify the total RNA from the adipose tissue samples for subsequent RNA sequencing on the HiSeq machine.

Task #3: Use whole transcriptome analysis in the CRC to examine expression of previously identified genes

Task #4: Investigate gender and patient subgroup differences in molecular response

Task #5: Discover new genetic influences on heart disease by profiling micro-RNAs and rare RNA transcripts
Tasks #3, #4, and #5 are using current patients in the Cardiovascular Risk Clinic. Fifty-three new participants entered the CRC program during the year. Blood samples were collected from a total of 324 participant-time points. In total, 6,196 aliquots for DNA, RNA, and plasma for biomarker assays were prepared and banked. DNA was isolated from 125 participants for molecular profiling on DMET™ SNP arrays; 49 DNA samples were assayed on the DMET™ SNP arrays and all samples had call rates >98%. RNA was isolated from 248 PAXgene tubes representing 150 participants in the CRC program; the range of concentrations was 5.7 ng/µl – 252.8 ng/µl.

Task #6: Develop systems biology approach to integrate various types of risk factor data

Task #6 will utilize the large-scale DNA and RNA sequence data generated in Tasks #1-#5, along with other CVD risk factor data collected in the Integrative Cardiac Health Program. To derive maximum information from the data, we are collaborating with scientists who have expertise in systems biology to integrate all of the different types of data. This approach will allow us to uncover inter-relationships and patterns within the data that may not be apparent when each modality is analyzed independently. Progress on this task will be made when we have sufficient DNA or RNA sequence data for analysis.

Discussion

The RNA concentrations resulting from isolation from blood or tissue are sufficient for down-stream applications. Using high quality RNA is a key element for successful microarray, RT-PCR, or RNA sequencing analyses. The RNA Integrity Number (RIN) was developed to remove individual interpretation in RNA quality control and to develop a classification of eukaryotic total RNA quality. The RIN numbers for all samples isolated for Tasks #2-#5 indicated that all samples are high quality and should yield quality RNA sequence.

Problems encountered during the year and plans to resolve them include:

1. Once the award was finalized in June 2012, generation of DNA and RNA sequence data could not begin until the HiSeq and MiSeq sequencing systems were in place. We encountered delays in contracting with local electrical firms to install the appropriate electrical lines, circuits, and outlets. Although ordered in July 2012, Illumina could not install the HiSeq machine and conduct training until early November 2012. During initial operation of the IlluminaCompute, the informatics system for production-scale sequencing with blade servers and modular storage devices, we encountered problems with proper function of the server system and storage of data. A subsequent service call to Illumina successfully resolved the server issues.

2. DNA contamination in RNA samples subjected to sequencing limited the amount of quality data. We believe the DNase was not functioning efficiently during the DNA removal step in the purification process. Experiments are ongoing to increase the efficiency of DNA removal and maximize collection of quality RNA sequence.

3. Three laboratory technicians in our group discontinued employment at WRI early in the third quarter of the award, leaving only one technician. Two of these technicians had been trained on the operation of the HiSeq and MiSeq systems. The shortage of trained personnel limited the amount of trouble-shooting, RNA isolations, and RNA sequencing data we were able to generate. We have advertised the positions through Windber Medical Center Human Resources, conducted numerous interviews, and recently hired two new Research Associates. However, both Associates will not begin employment until the next quarter. In addition, both Associates will need training on how to operate the sophisticated

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HiSeq and MiSeq sequencing systems. We are currently evaluating our options for how best to secure training for the new personnel.

4. For Task #2, one issue encountered during the year involves LAGB patients not showing up for their scheduled follow-up examinations. This absenteeism reduces the number of follow-up examinations from which we have physiological data and blood samples for molecular analysis. We will encourage patients to keep their follow-up appointments.

4. **KEY RESEARCH ACCOMPLISHMENTS:** Nothing to report.

5. **CONCLUSION:** During the next year, our main focus will be on securing training for the new Research Associates on the HiSeq and MiSeq sequencing systems, trouble-shooting the DNA contamination issue, and generating quality RNA and DNA sequence data. With sufficient preliminary data, we will begin integrating the various types of data and begin preparing abstracts for presentation and publication.

6. **PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:** Nothing to report.

7. **INVENTIONS, PATENTS AND LICENSES:** Nothing to report.

8. **REPORTABLE OUTCOMES:** Nothing to report.

9. **OTHER ACHIEVEMENTS:** Nothing to report.

10. **REFERENCES:** Nothing to report.

11. **APPENDICES:** Nothing to report.